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ORIGINAL ARTICLE

Protein substitution to produce a processed cheese with high branched-chain amino acids of medical and genetic importance

Howida A.M. El-Shazly [a,*], Rizk A. Awad [b], Ehab A.Y. Essawy [a], Tarek M. Kamal [c], Wafaa M. Salama [a]

[a] *Dairy Research Department, Food Technology Research Institute, Agricultural Research Centre, Egypt*
[b] *Food Science Department, Faculty of Agriculture, Ain Shams University, Egypt*
[c] *Genetics Unit, Pediatrics Department, Faculty of Medicine, Ain Shams University, Egypt*

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KEYWORDS

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Liver;
Brain;
Phenylalanine;
BALB/c mice

Abstract

Background: The most important metabolic impairment in patients with advanced liver disease is characterized by low levels of circulating branched chain amino acids (BCAAs). The etiology of such abnormal amino acid metabolism is multifactorial including protein restricted diet or inadequate nutritional intake as in protein energy malnutrition. Multiple studies report the beneficial effects of BCAAs supplementation to improve plasma amino acids imbalance, several neurologic diseases, protein energy malnutrition, and subsequently the survival rate of cirrhotic patients.

Methods: In the present study we used a protein substitution technique to synthesize a new processed cheese as a dairy source rich in BCAAs, with low phenylalanine content manufactured from Ras cheese, kariesh cheese, butter oil and phenylalanine-free milk. Chemical composition, amino acids analysis, rheological properties and sensory evaluation were done to all of the cheese samples. L-Phenylalanine was selected to induce hepatic and brain affections in Begg Albino strain c (BALB/c) mice model. Effect of 2.5%, 5% and 10% protein-replacement cheese formulas was evaluated among mice groups including histopathological sections of the liver and brain; colorimetric determination

* Corresponding author. Tel.: +2 012 1372092.
E-mail address: hammaetl@yahoo.com (H.A.M. El-Shazly).

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for liver enzymes; serum total and differential cholesterol profile, serum albumin, globulin and total protein along with phenylalanine levels determinations.

Results: Analysis of the processed cheese sample with 10% protein substitution revealed that the protein content was 7.42 mg/g (about 50% of the content in the standard processed cheese) while fat content, acidity and moisture were nearly the same. The sensory score for all the formulas ranged from 79–88. Highest content of BCAAs along with least phenylalanine content was attained in the processed cheese with 10% protein substitution. Weight of mice fed on different substitution formulas ranged from 22.8 ± 2.2–24.66 ± 2.5 g compared with 17.8 ± 1.9 g in the untreated diseased mice ($P < 0.05$). Serum phenylalanine was 1.822 ± 0.42 mg/dl in the mice fed on 10% protein substitution formula compared to 6.2 ± 1.32 mg/dl in the untreated mice ($P < 0.01$). There was a highly significant difference ($P < 0.01$) between untreated mice and mice fed on 10% substitution cheese formula as regards the serum protein, Aspartate Transaminase (AST) and Alanine Transaminase (ALT). The improvement in histopathological findings was more apparent in the mice fed on 10% formula cheese.

Conclusion: The manufactured processed cheese with 10% protein substitution was proved to have a more nutritional therapeutic potential that can help in the implementation of dietary management in many medical and genetic disorders with liver and brain affections.

1. Introduction

The restriction of dietary protein has long been considered a mainstay in the therapy of hepatic encephalopathy. More recently, it has been recognized that protein – energy malnutrition is frequent in advanced liver disease and may adversely affect the patient's outcome [1]. Cirrhosis represents the final stage of many chronic liver diseases and is associated with more or less pronounced hyponutrition. The most important metabolic impairment in patients with advanced liver disease is the change in amino acid metabolism. The plasma levels of branched chain amino acids (BCAA) are decreased [2]. Multiple studies report the beneficial effects of branched-chain amino acids supplementation, including improved metabolic profiles as measured by protein sparing and clinical improvement of hepatic encephalopathy. Increasing evidences show that the oxidative stress is involved in a large number of diseases as arteriosclerosis, diabetes, phenylketonuria, neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease; cancer and aging among others [3]. Many of the features of BCAAs metabolism in tumor-bearing state are similar to other disease states that feature involuntary weight loss and skeletal muscle atrophy. These states are generally characterized by altered BCAAs availability (low BCAAs intake, elevated rate of BCAAs oxidation, and gluconeogenesis), which are concurrent with the activation of proteolysis and the suppression of protein synthesis in skeletal muscles. These features are the basis of suggestions for dietary supplementation with BCAA or their metabolites [4]. In patients with phenylketonuria, a higher dosage of the protein substitute appeared to contribute to lower blood phenylalanine concentrations [5] with the branched-chain amino acids (BCAAs) influencing brain function by modifying large neutral amino acid (LNAA) transport at the blood–brain barrier. The chronic uses of BCAA supplementations have been evaluated in Phenylketonuria (PKU) as either an adjunct to or substitute for a low-phenylalanine diet .The nature of amino acid composition of dietary proteins contributes to cerebral function and play a major role in affecting the brain in neurodegenerative disorders [6]. In individuals with disease states like phenylketonuria, hepatic encephalopathy, bipolar disorder, and other neurological diseases, BCAAs supplementations have been given to diminish or to retard the progression of central nervous system functional symptoms. In addition, oral BCAAs supplements have been examined as a treatment for patients with tardive dykinesia [7], amyotrophic lateral sclerosis [8], spinocerebellar degeneration [9] and X-linked adrenoleukodystrophy [10].

Since BCAAs cannot be synthesized de novo, they must be obtained from the diet for protein synthesis [11].

In the present study we tried to use a protein substitution technique to synthesize a new processed cheese rich in branched-chain amino acids, with low phenylalanine content and improved protein quality. This cheese can be of nutritional importance as a supplement in some medical and genetic disorders in liver and brain affection.

2. Materials and methods

Phenylalanine-free commercial milk formula MD mil MD ™PKU.O: was obtained from Liptis Pharmaceuticals®, USA.

Chemicals:

1. L-Phenylalnine for mutagenesis was obtained from Aldrich-Chemie GmbH & Co™ (Germany).
2. Kits for total cholesterol, LDL, triglycerides, total protein, albumin, Aspartate Transaminase (AST) and Alanine Transaminase (ALT) were obtained from Biodiagnostic™, Giza, Egypt. All other chemicals used through analysis were obtained from (Sigma Chemical Co., P.O. Box 14508, St. Louis, USA).

Experimental animals: To avoid any potential confounding difference due to sex and age, only adult female Begg Albino strain c (BALB/c) mice were obtained from Research Institute of Ophthalmology, Giza, Egypt.

2.1. Processed cheese manufacture

A suitable amount as described in Table 1 of mature Ras cheese, Karish cheese, butter oil, emulsifying salt and the

Table 1 Amount of the ingredients (g) used in manufacture of processed cheese.

Component	2.5% Protein substitution in cheese	5% Protein substitution in cheese	10% Protein substitution in cheese	Cheese without protein substitution
Ras cheese	350	312.5	225	375
Kariesh cheese	462.5	387.5	250	562.5
phenylalanine-free milk	31.25	62.5	125	–
Emulsified salt	31.25	31.25	31.25	31.25
Fat	123.875	134.87	162.25	115
Water	251.125	321.375	456.5	166.25
pH	5.8	5.8	6.2	5.8

phenylalanine-free milk formula were added at the rate of 2.5%, 5%, 10% consecutively in a laboratory processing kettle. Control treatment was adjusted to have the same composition without adding the phenylalanine-free milk formula. The mixture was cooked for 10 min at 85–90 °C using indirect steam at pressure 2–2.5 kg/cm^2. The melted processed cheese was poured into wide mouth glass jars and capped directly after filling. The resultant cheese was analyzed when fresh and stored in the refrigerator (5 ± 2 °C) during the animal experimental study.

Chemical analysis: Moisture, protein, ash, fat and titratable acidity of the cheese were determined according to AOAC [12]. The pH values were measured using a digital pH meter model SA 720 (Orion, USA). Amino acid analysis by HCl hydrolysis was performed when required, previous to amino acid derivatization; using 6 N HCl for 21 h at 110 °C in sealed glass ampoules.

Rheological properties: Oil separation index of the processed cheese was determined as described by Thomas [13]. The diameter of the separated oil was measured in millimeter and used as an oil separation index. Cheese meltability was measured as described by Olson and Price, [14] with the modification of Rayan et al. [15] using the meltability test apparatus. Cheese firmness was measured using a penetrometer cone and was adjusted to touch the sample for 5 s. The penetration depth was recorded in units of 0.1 mm. Penetrometer reading is related inversely to firmness of the cheese spread [16].

Sensory evaluation: Samples of processed cheese were subjected to sensory evaluation according to the scheme of Mayer [17] by 15 regular scoring panel.

Animal experiment: Thirty-three BALB/c mice 3 weeks old were raised in The Research Institute of Ophthalmology, Giza, Egypt. The mice were given a standard diet as described by Campbell [18] for 1 week (adaptation period) under normal healthy conditions. At the end of the adaptation period, mice reached an approximate weight of 24–27 g (average 25.2 ± 1.2 g). The animals were housed in cages at room temperature (about 25 ± 5 °C).

Design of experiment: After the adaptation period, three mice were randomly chosen as a control for normal mice then weighed. Blood samples were withdrawn from retro bulbar venous plexus of each mouse according to the procedure of Shermer [19], then the mice were slaughtered and liver and brain, were excised, weighed and stored until biochemical analysis. The other thirty mice were fed on a mutagenic diet consisting of standard diet plus 0.3% phenylalanine (as an inhibitor to phenylalanine hydroxylase enzyme) to induce hyperphenylalaninemia according to Schott et al. [20] and Austic et al. [21] for additional 2 weeks. Five mice were randomly selected and sacrificed in order to do biochemical analysis and histopathological study to the liver and brain after mutagenesis (at zero time). The other 25 diseased mice were then divided into five groups according to the following scheme:

Group (1) Fed on the processed cheese with 2.5%protein substitution for 3 weeks.
Group (2) Fed on the processed cheese with 5% protein substitution for 3 weeks.
Group (3) Fed on processed cheese with 10% protein substitution for 3 weeks.
Group (4) Fed on processed cheese without protein substitution for 3 weeks.
Group (5) Fed on the phenylalanine-free formula milk alone for 3 weeks.

Phenylalanine level: Phenylalanine level was estimated in blood of mice every week (51 samples) according to McCaman and Robbins [22] using HPLC equipment Wallac fluorometer system™ (Perkin-Elmer Life Science Inc., Norton, OH, USA).

Histological sections: Animals were slaughtered. Liver and brain were quickly removed and immediately potted in chloroform. Liver and brain sections were prepared, stained and viewed as described by Mahfouz et al. [23].

Serum analysis: The obtained serum from BALB/c mice was analyzed for total cholesterol, Low density lipoprotein cholesterol (LDL) and triglycerides using enzymatic colorimetric method according to Trinder [24] and as described by Lin et al. [25]. Total protein content was determined using the method of Henery [26]. Albumin content was determined using method of Doumas et al. [27].

Colorimetric method was used to determine AST and ALT testing for liver function according to Reitman and Frankel [28].

Statistical analysis: Duncan and Dunnett *t*-test were used to determine statistical significance among the different tested mice groups. The results were presented as the mean ± the mean; P values <0.05 were considered significant and $P < 0.01$ highly significant.

3. Results

The protein content was reduced to 7.427 mg/g in the 10% protein substitution sample (about 49.5% of the control cheese without protein substitution). Moisture content was kept constant (at 60%) and the fat content ranged between 16.5% and 17% in all of the processed cheese formulas with various

Table 2 Chemical analysis of the manufactured processed cheese.

Constituent	2.5% Protein substitution in cheese	5% Protein substitution in Cheese	10% Protein substitution in Cheese	Control cheese without protein substitution
Acidity (%)	2.1	1.7	1.2	1.1
Protein (mg/g)	11.469	9.7	7.427	15
Ash (%)	4.16	4.05	3.385	4.68
Moisture (%)	60	60	60	60
Fat (%)	16.5	17	17	16.5

grades of protein substitution (2.5%, 5% and 10%) compared to the control cheese without protein substitution (Table 2).

The rheological properties (meltability, penetration and oil separation) of processed cheese containing the phenylalanine-free milk formula were higher than those of the control cheese without protein substitution. The melting value was increased with increasing percentage of protein substitution being highest in 10% substitution sample. The lowest penetration value was found in control cheese without protein substitution, while the highest was noticed with protein substitution treatment (10%) followed by 5% then 2.5%. This means that control treatment was the firmest while 10% substitution was the softest. Sensory evaluation of the processed cheese indicate that cheese control without protein substitution ranked the highest scores for different sensory attributes and highly accepted to panelists, followed by the other treatments. Addition of 5% protein substitution to cheese improved the flavor, body and texture as well as cheese appearance of the resultant spreads. Increasing the ratio of added protein substitution led to less acceptable products being lowest at 10% cheese protein substitution (Table 3 and Diagram 1).

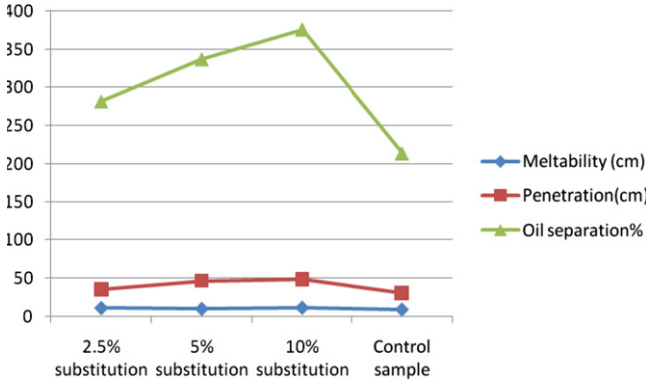

Diagram 1

The highest content of branched chain amino acids (valine, leucine and Isoleucine) was attained in the processed cheese with 10% protein substitution along with 71.96% reduction in the content of phenylalanine (309.78 mg/g) as compared to 1104.56 mg/g in the processed cheese without protein substitution (Table 4).

There was a highly significant reduction in the mean value of phenylalanine ($P < 0.01$) as well as significant increase in weight ($P < 0.05$) among the mice groups at zero time before treatment and those mice groups that received treatment formulas as compared with both of the positive control and diseased mice groups. The highest reduction in phenylalanine was attained with the phenylalanine-free milk (Table 5 and Diagram 2).

There was a significant improvement in the mean values of total protein, serum albumin and liver enzymes among diseased mice groups receiving treatment formulas with better values obtained among those mice fed on 10% protein substitution cheese as compared with positive control group. On the other hand, there was no significant difference as regards level of total cholesterol, LDL-C and triglycerides ($P > 0.05$) (Table 6).

Figs. 1–8 and Table 7 show the histopathological findings in (a) the brain and (b) liver of BALB/c mice before and after feeding on 2.5%, 5% and 10% protein substitution cheese as compared to low-protein formula feeding group.

Table 3 Sensory and rheological evaluation of the processed cheese samples.

Evaluation	2.5% Protein substitution in cheese	5% Protein substitution in cheese	10% Protein substitution in cheese	Control cheese without protein substitution
I-sensory				
Flavor (40)	35	36	31	38
Body (40)	35	36	32	37
Appearance (20)	18	19	16	19
Total score	88	91	79	94
II-rheological				
Meltability (cm)	10	11.1	11.5	9.2
Penetration (ml)	36	47	49	31
Oil separation (%)	282.3	337.5	376.4	214.4

Table 4 Amino acid analysis of different processed cheese samples vs cheese without protein substitution (mg/g).

Amino acid	Control cheese without protein substitution	2.5% Protein substitution in cheese	5% Protein substitution in cheese	10% Protein substitution in cheese
Aspartic	1113.04	802.72	883.44	954
Threonine	634.08	390	424.64	456.36
Serine	831.6	484.4	473.04	637.68
Glutamic	2805.28	2166.08	2393.2	2350.4
Glycine	219.12	143.28	155.04	167.76
Alanine	562.56	345.28	386.64	403.04
Valine	768.72	489.36	588.48	1348.08
Methionine	445.2	49.52	100.64	55.6
Isoleucine	725.92	486.32	498.32	1114.88
Leucine	1584	1114.32	1138.4	2458.08
Tyrosine	1110.08	690.96	783.84	788.24
Phenylalanine	1104.56	840.4	624.4	309.68
Histidine	764.64	403.2	505.12	570.88
Lysine	1226.88	753.76	866	880
NH_4	1715.2	1454.4	1585.76	1534.56
Arginine	579.92	386	385.28	448.32
Proline	5900.5	3705.049	4250.239	245.864

Table 5 Mean values of weight and phenylalanine levels in all of the mice groups before and after feeding on different cheese substitution samples.

Value	0-Time	Diseased for 2 weeks	2.5% Substitution in cheese	5% Protein substitution in cheese	10% Protein substitution in cheese	+ve Control	Ph.al. free milk	*P*-value
Weight (g)	25.2 ± 1.2	19.433 ± 3.5	22.8 ± 2.2	23.8 ± 3.1	24.66 ± 2.5	17.8 ± 1.9	22.2 ± 2.9	$P < 0.05$
Ph.al. (mg/d)	2.274 ± 0.84	5.79 ± 0.98	3.314 ± 1.1	2.166 ± 0.8	1.822 ± 0.42	6.2 ± 1.32	1.291 ± 0.3	$P < 0.01$

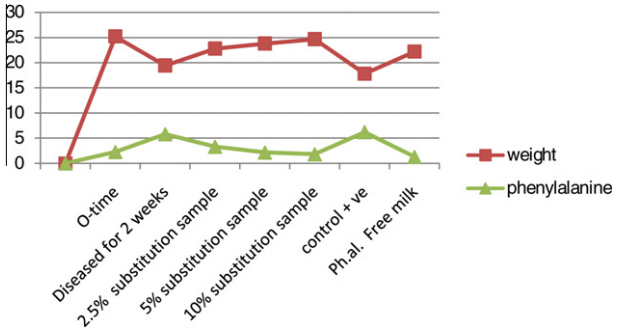

Diagram 2

4. Discussion

The aim of the present study was to synthesize a new processed cheese as a dairy product rich in branched-chain amino acids and high quality protein for patients with the liver disease.

Mesejio et al. [2] recommended the use of branched-chain amino acids supplementation derived from either diets with vegetable proteins or dairy proteins to slow the progression of liver disease and improve survival of patients with compensated cirrhosis and encephalopathy. The branched-chain amino acids (BCAAs) are required for protein synthesis and neurotransmitter synthesis. BCAAs cannot be synthesized de novo and must, therefore, be obtained from the diet for protein synthesis. Leucine is of special interest because it promotes protein synthesis, inhibits protein degradation, and stimulates insulin release. As a result of these actions of leucine, BCAAs

Table 6 Mean values for biochemical findings in all of the mice groups before and after feeding on the cheese samples.

Findings	0-Time	Diseased for 2 weeks	2.5% Protein substitution in cheese	5% Protein substitution in cheese	10% Protein substitution in cheese	+ve Control	Ph.al.Free milk	*P*-value
Total protein (g/dl)	5.986 ± 1.11	3.2 ± 0.52	3. 6 ± 0.41	4.9 ± 0.63	5.2 ± 0.641	1.96 ± 0.32	3.8 ± 0.784	$P < 0.05$
Albumin (g/dl)	2.7 ± 0.96	1.44 ± 0.61	1.72 ± 0.56	2.25 ± 0.88	2.4 ± 0.48	0.9 ± 0.08	1.63 ± 0.43	$P < 0.05$
Total cholesterol	94.9 ± 7.2	85.9 ± 9.8	88.1 ± 6.9	89 ± 10.1	96.2 ± 8.87	83 ± 7.9	79 ± 8.2	$P > 0.05$
LDL-C	34.5 ± 3.5	32.1 ± 4.66	33.21 ± 4.9	33.41 ± 3.2	34 ± 2.87	38 ± 4.66	30 ± 1.99	$P > 0.05$
Triglycerides	116.8 ± 10.1	152 ± 8.2	121 ± 9.98	118.2 ± 8.91	119.1 ± 7.93	201 ± 12.97	148 ± 13.22	$P > 0.05$
AST(U/ml)	41.7 ± 2.2	90 ± 3.32	45.2 ± 2.11	44.3 ± 3.49	42 ± 1.88	95 ± 6.1	39 ± 2.0	$P < 0.01$
ALT (U/ml)	35 ± .99	83 ± 1.9	48 ± 3.0	38.7 ± 3.2	37 ± 2.98	87 ± 5.4	37.2 ± 2.8	$P < 0.05$

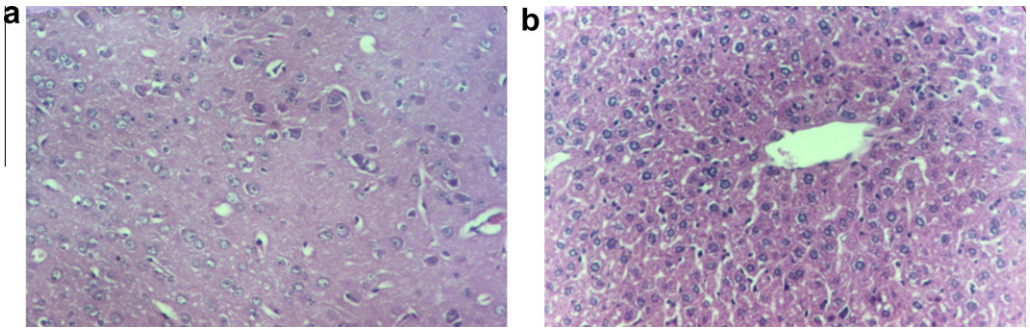

Figure 1 (a) The brain histopathology: brain of normal BALB/c mice showing no histopathological changes (stained with H and E) (X200). (b) The liver histopathology: liver of control normal BALB/c mice having normal histological structure of hepatic lobule (stained with H and E) (X200).

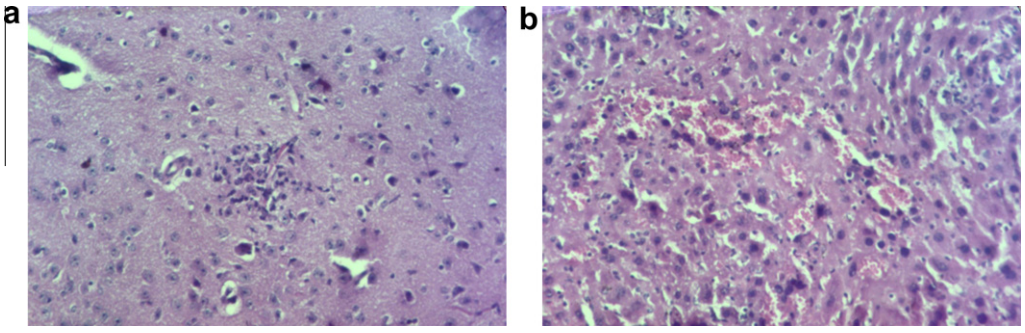

Figure 2 (a) The brain histopathology: brain from affected BALB/c mice group at zero time after mutagenesis showing necrosis of neurons associated with focal gliosis (stained with H and E) (X200). (b) The liver histopathology: liver from affected BALB/c mice group at zero time after mutagenesis showing necrosis of hepatocytes, pyknosis of their nuclei and focal hepatic hemorrhage (stained with H and E) (X200).

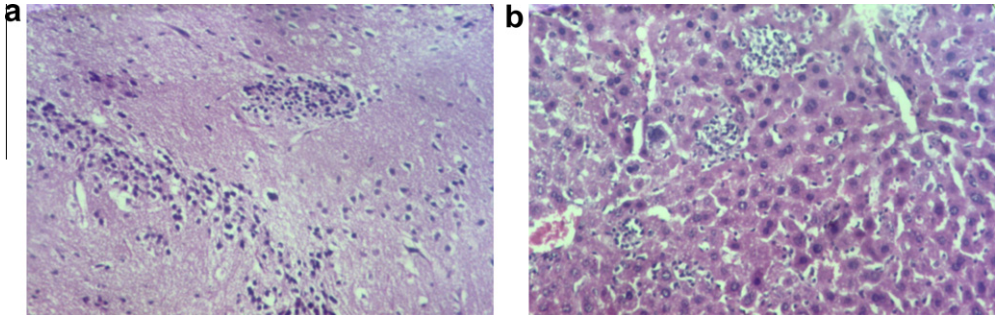

Figure 3 (a) The brain histopathology: brain of BALB/c mice from + ve control group showing necrosis and focal gliosis (stained with H and E) (X200). (b) The liver histopathology: liver of BALB/c mice from + ve control group showing multifocal hepatic necrosis associated with leucocytic cells infiltration (stained with H and E) (X200).

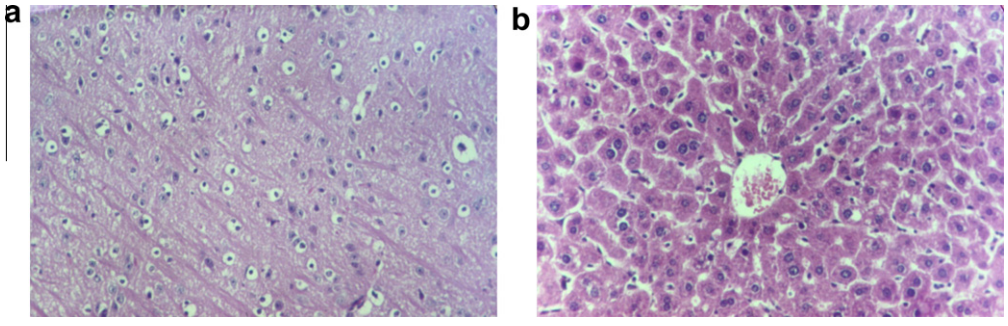

Figure 4 (a) The brain histopathology: brain of BALB/c mice from group 1 showing neuronal edema (stained with H and E) (X200). (b) The liver histopathology: liver of BALB/c mice from group 1 showing hemorrhage and necrosis of sporadic hepatocytes (stained with H and E) (X200).

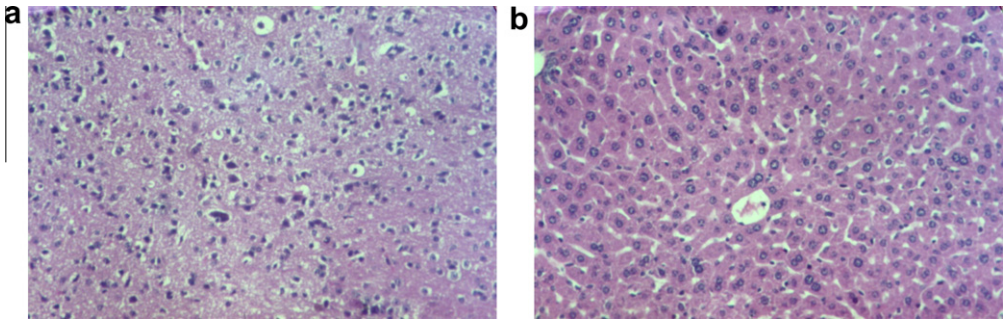

Figure 5 (a) The brain histopathology: brain of BALB/c mice from group 2 showing necrosis of neurons associated with diffuse gliosis (stained with H and E) (X200). (b) The liver histopathology: liver of BALB/c mice from group 2 showing no histopathological changes (stained with H and E) (X200).

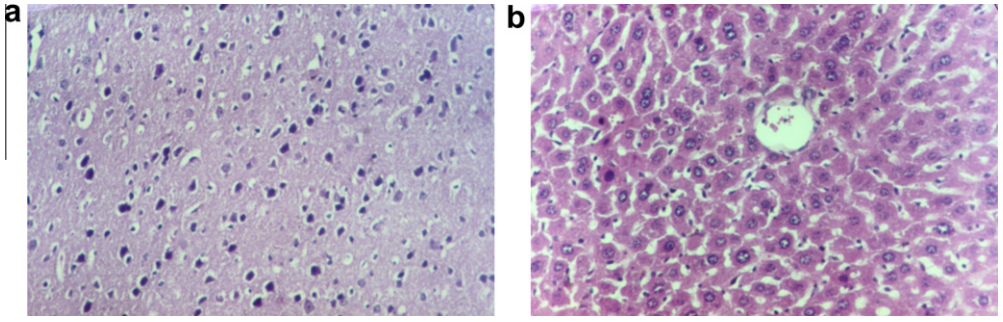

Figure 6 (a) The brain histopathology: brain of BALB/c mice from group 3 showing pyknosis of neurons (stained with H and E) (X200). (b) The liver histopathology: liver of BALB/c mice from group3 revealed sporadic necrosis of hepatocytes with resorption of hemorrhage, restoration of architecture of lobules, nuclei and inhibition of inflammation in the hepatocytes (stained with H and E) (X200).

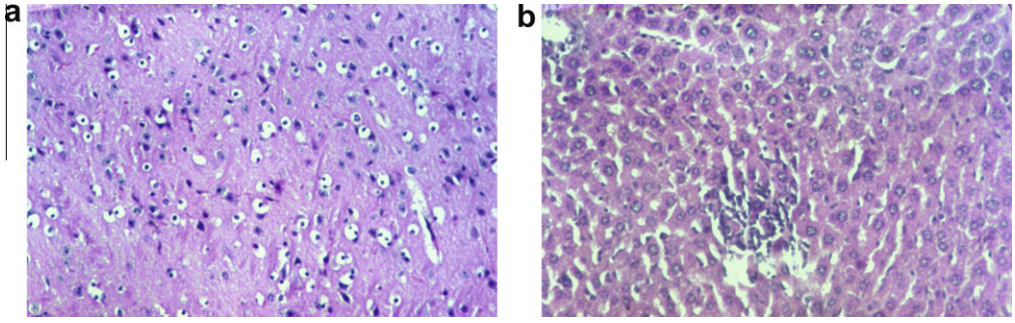

Figure 7 (a) The brain histopathology: brain of BALB/c mice from group 4 showing cellular edema and diffuse gliosis (stained with H and E) (X200). (b) The liver histopathology: liver of BALB/c mice from group 4 showing focal hepatic necrosis associated with leucocytic cells infiltration (stained with H and E) (X200).

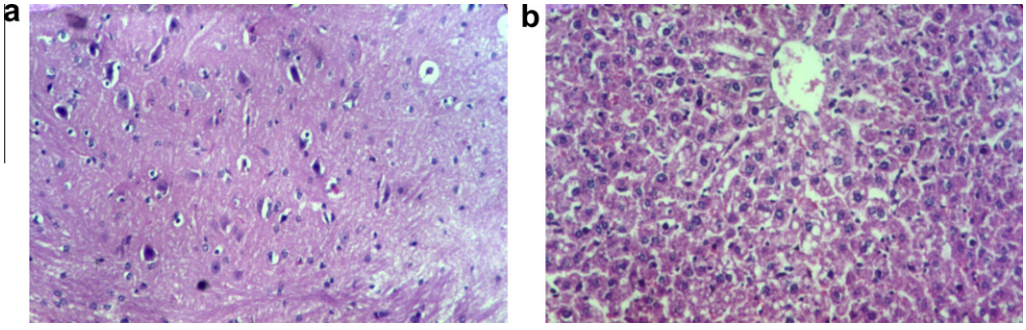

Figure 8 (a) The brain histopathology: brain of BALB/c mice from group 5 showing neuronal degeneration and neuronophagia (stained with H and E) (X200). (b) The liver histopathology: liver of BALB/c mice from group 5 showing vacuolar degeneration of hepatocytes (stained with H and E) (X200).

Table 7 Histopathological findings in the brain and liver of mice groups before and after feeding on the protein substitution cheese compared to the group fed on the low-protein phenylalanine-free formula.

Group	Findings
I-liver	
Normal mice	Normal hepatic lobules
Affected mice 0-time	Necrosis of hepatocytes/pyknosis of nuclei/focal hemorrhage, multifocal necrosis with leukocytic infiltration
Group 1	Necrosis of sporadic hepatocytes
Group 2	No remarkable changes from group 1
Group 3	Sporadic necrosis/resorption of hemorrhage /restoration of hepatocytes architecture.
Group 4	Focal necrosis with leukocytic infiltration
Phe.al. free milk	Vacuolar degeneration of hepatocytes.
II-brain	
Normal mice	Normal histological findings of brain cells
Affected mice 0-time	Necrosis of neurons/focal gliosis
Group 1	Edema of neurons/mild to moderate gliotic cells
Group 2	Necrosis of neurons/diffuse gliosis
Group 3	Pyknosis of neurons
Group 4	Cellular edema
Phe.al. free milk	Neuronal degeneration neuronophagia

Phe.al. = phenylalanine.

have therapeutic potential, because they spare lean body mass during weight loss, promote wound healing, and decrease muscle wasting with aging [29]. Altered amino acids metabolism is a hallmark of liver diseases and liver cirrhosis, characterized by low levels of circulating BCAAs and elevated levels of circulating aromatic amino acids [11]. These abnormalities are known to induce hepatic encephalopathy and muscular waste. The etiology of such abnormal amino acids metabolism is multifactorial including protein restricted diet or inadequate nutritional intake as in protein energy malnutrition. Therefore, BCCAs supplementation seems to be useful to improve plasma amino acids imbalance, protein energy malnutrition, and subsequently survival rate of cirrhotic patients [30].

In the present study, processed cheese was selected as a possible nutritional source of branched-chain amino acids (BCAAs) since it is becoming increasingly the popular cheese worldwide with continuous increase in its production [16]. In Egypt, processed cheese has recently become a popular cheese type. In 1997, it represented about a quarter of the total cheese imports of 120,000 tons and its production either spreadable or block type has reached about 10,000 tons in the governmental and private sectors [31]. Using a palatable formulation, the investigators demonstrated that long-term BCAAs supplementation is associated with decreased frequency of hepatic failure, however, cost and palatability may limit applicability of this type of treatment modalities. Therefore, processed cheese – as a significant source of protein and other nutrients – represents an attractive dairy product for several consumers especially for children [16].

In the present work, the selection of a phenylalanine-free, low-protein formula to be used in the production of this processed cheese may make it potentially useful for use in conditions needing low protein and or low-phenylalanine in diet.

The low-phenylalanine diet is maintained relatively easily during periods of rapid growth in infancy and early childhood. As somatic growth slows and the utilization of phenylalanine decreases, it becomes increasingly difficult to achieve and maintain low serum phenylalanine [32]. Diet for life has been difficult because strict dietary regimens leads to erosion of dietary adherence as children get older. This has caused reassessment of treatment strategies and prompted the National Institute of Health Recommendations for treatment guidelines [33]. Lower levels of BCAAs (along with large neutral amino acids) have been reported in patients with PKU and PKU mice [34]. Motor and cognitive functioning improved with addition of Valine, Isoleucine and leucine supplements to the low-phenylalanine diet [32].

The amino acids analysis of the processed cheese with 10% protein substitution revealed high content of Valine (1348.08 mg/g), Isoleucine (1114.88 mg/g) and Leucine (2458.08 mg/g) compared to the 2.5% and the 5% substitution products. The ratio between Leucine and Isoleucine 2.2:1 while it is 1:1.2 between Isoleucine and Valine.

This is in consistency with Baker [35] who stated that most studies involving pharmacologic BCAAs administration have used mixtures containing 50–100% more Leucine than Isoleucine, and generally slightly more Valine than Isoleucine. The requirement ratios of the estimated average requirement (EAR) estimates of the Dietary Recommended Intake (DRI) Committee (2002) are 2.3 (Leucine):1.0 (Isoleucine):1.3 (Valine). The relation of one BCAAs to another (i.e., BCAAs ratios) is of interest since pharmacologic BCAAs dosing for muscle endurance or for other purposes generally has involved administration of all three BCAAs [36,37]. The Daily Recommended Intake (DRI) Committee [38] arrived at the estimated average requirement (EAR) of 34, 15, and 19 mg/kg/day for Leucine, Isoleucine, and Valine, respectively. The University of Toronto group also reported that the BCAA requirement of young children was 48% higher than the Daily Recommended Intake (DRI) Committee recommendation [39]. No definitive graded dosing studies with humans exist, but there are a few instances where BCAAs dosing of 2–3 times the EAR have been reported. Marchesini [40] treated 20 chronic hepatic encephalopathy patients for 6 months, with an enteral supplement providing 240 mg/kg/day of BCAA. This report contains no reference to either toxicity or adverse effects. Patients with sepsis, stress, or injury have likewise been treated

with parenteral solutions containing up to 50% of the AA nitrogen as BCAAs, with no apparent adverse side effects. It appears based on both animal and human studies that BCAAs are among the best tolerated of all amino acids when intakes well above the requirement are consumed. Animal graded dosing studies together with human single dosing studies suggest that BCAA intake levels of at least three times the requirement level are well tolerated [41,35].

In the present work, low-protein formula was utilized to make different grades of protein substitutions (2.5%, 5% and 10% protein substitution in the processed cheese) then their effects were compared among the different affected and control mice groups. Significantly higher improvement was observed in blood protein level among mice fed on the cheese with 10% protein substitution.

Several investigators have suggested that high concentrations of certain amino acids in plasma or brain may provide a signal for reduction of protein consumption [42]. Peters and Harper [43] observed a strong correlation between protein intake of rats and concentrations of branched-chain amino acids in plasma and brain and suggested that changes in plasma or brain BCAA concentrations might serve as a signal for a change in protein intake. On the other hand, Anderson and Associates [44] stated that elevated plasma and brain BCAA concentrations were not associated consistently with altered protein selection or changes in food intake. The most important dietary factor impacting BCAA tolerance levels is protein level. The human maintenance BCAA requirement estimates range from 10.3% to 22% of the maintenance protein requirement [35]. The current recommended dietary allowance (RDA) for protein is 0.8 g/kg/d for adults or about 56 g protein /d for a 70 kg person. The daily intake of the BCAAs in a 70-kg person consuming the RDA for protein would thus be 8.4–11.2 g. [45]. MacDonalds and Associates [5] concluded that in PKU patients, a higher dosage of the protein substitute appeared to contribute to lower phenylalanine concentrations but it did have a variable and individual impact and may have been influenced by the carbohydrates and fat content of the protein substitute.

In the present study, we used L-phenylalanine in BALB/c mice model to induce elevation in liver enzymes, hyperphenylalaninemia, beside affection of the growth and liver pathology.

Schott et al. [20] stated that activity of liver phenylalanine hydroxylase was decreased in animals receiving phenylalanine. In animals treated with phenylalanine alone concentration of serum phenylalanine was raised about 7-folds and triglyceride content of the liver slightly above the control. The most obvious side effect was growth retardation and fatty liver [20]. The liver responds to changes in nutrient availability by initiating a number of stress signaling pathways [46]. Cell damage by oxygen radicals and lipid peroxidation play a crucial and causative role in the pathogenesis of several acute and chronic diseases, such as cancer, aging, atherosclerosis and liver injury [47]. Hepatic illnesses in general provoke concomitant increases of Alanine Transaminase (ALT) and Aspartate Transaminase (AST) levels and increase in serum ALT activity are rarely observed in conditions other than liver illness [48]. Studies in humans showed that oxidative stress occurs also in patients with phenylketonuria probably contributing to the neurological damage in this disorder. Exposure to high phenylalanine concentrations for a short or long time results in a reduction of enzymatic and non-enzymatic antioxidant defenses, whereas protein and lipid oxidative damage only occurs in patients with late diagnosis [49].

The present study showed decrease in total serum protein, albumin and cholesterol while LDL-C and triglycerides were insignificantly elevated above the control after induction of hyperphenylalaninemia ($P > 0.05$). Best normalization and approximation to normal control values of protein ($P < 0.05$) and cholesterol profile ($P > 0.05$) was obtained in the mice fed on 10% substitution formula compared to the other formulas.

Colomé et al. [50] stated that serum cholesterol concentrations were significantly lower in PKU patients compared with inborn errors of metabolism patients (whose cholesterol daily intake was similar to those of PKU patients), children with hyperphenylalaninemia and the control group. A negative correlation was observed between cholesterol and phenylalanine concentrations in the PKU patients supporting the hypothesis of a relationship between high plasma phenylalanine levels and an inhibition of cholesterogenesis, although the low cholesterol intake of the special diets may also decrease serum cholesterol values. Furthermore, blood cholesterol levels of these children were low in comparison with the levels of healthy children of the same age, but the triglyceride levels were higher as a result of the special diets containing a large amount of carbohydrates [51]. Previous reports have suggested that elevated levels of phenylalanine inhibit cholesterol [52]. However, Terry et al. [53] added that lipoprotein abnormalities noted between unrelated subjects with and without phenylketonuria are rather due to a genetic predisposition of the population with phenylketonuria toward lower serum lipid concentrations.

The use of 10% substitution formula with more content of branched-chain amino acids resulted in a 26.89% gain in weight of mice compared to 22.24% among mice fed 5% protein substitution cheese and only 17.3% in the group fed 2.5% protein substitution cheese formula.

An anabolic effect of leucine and the branched-chain amino acids was found on reduction of muscle protein breakdown in humans [54]. Similar studies in liver showed stimulation of protein synthesis by leucine and a reduction in protein break-down [55]. Ingestion of BCAAs increases their concentration in plasma. This may reduce the uptake of tryptophan by the brain and also 5-HT synthesis and thereby delay fatigue. Accordingly, when BCAAs were supplied to human subjects during a standardized cycle ergometer exercise (to measure the amount of work and energy expenditure done by the muscles over a period of time) their ratings of perceived exertion and mental fatigue were reduced [56]. Cangiano et al. [57] hypothesized that the oral administration of BCAAs to cancer patients with anorexia would lead to decreased brain tryptophan concentrations and reduced serotoninergic activity, eventually resulting in an improvement of food intake and, therefore, BCAAs may be used safely to improve caloric intake in cancer patients with anorexia. The BCAAs are central in the maintenance of lean body mass and regulation of skeletal muscle protein metabolism [4].

In the present study, there was a reduction in the serum phenylalanine by 68.5% in the mice group fed on cheese with 10% protein substitution compared to 77.7% in group fed on phenylalanine free formula with less reductions in the mice groups fed 5% (62.5%) and 2.5% protein substitution cheese (42.7%), respectively.

Branched-chain amino acids (BCAAs) influence brain function by modifying large, neutral amino acid (LNAA) transport at the blood–brain barrier. Transport is shared by several LNAAs, notably the BCAAs and the aromatic amino acids (ArAAs), and is competitive [58]. The use of BCAAs dietary "supplements" has been examined as a means to promote reductions (or further reductions) in brain phenylalanine concentrations. The notion is that by elevating plasma concentrations of the BCAAs, brain phenylalanine (PHE) uptake can be diminished (or further diminished), thereby producing reductions in brain phenylalanine concentrations and a beneficial effect to brain function. The chronic use of BCAA supplements has been evaluated in PKU subjects as either an adjunct to or substitute for a low-PHE diet (e.g., in patients unable to maintain the rather restrictive low-PHE diet) for up to 6 weeks in four divided daily doses totaling 500 mg/kg/d. This treatment significantly elevated plasma and cerebrospinal fluid (CSF) concentrations of the BCAAs and reduced CSF concentrations of both PHE and TYR in adolescents and adults [59,60]. This treatment paradigm was associated with no adverse effects, and improvements in some cognitive functions were noted [32]. The highest doses of BCAAs that have been administered chronically to humans have been to PKU [up to 35 g/d] [59] and in patients with mania [up to 60 g/d] [32].

The histopathological findings were improved more in the liver and brain sections of mice from group 3 (fed on cheese with 10% protein substitution) than the remaining mice groups as revealed in the liver by the resorption of hemorrhage, restoration of architecture of lobules and inhibition of inflammation in the hepatocytes and in the brain by the regression of inflammatory and cellular edema together with areas showing signs of necrosis.

Infusion of BCAAs in patients with hepatic cirrhosis blocked the abnormal uptake of tyrosine by the brain [61]. Furthermore, administering valine to rats prevented the exercise-induced 5-HT release in the ventral hippocampus during and after exercise [62]. Both studies indicate that elevating the plasma concentration of BCAAs (or valine) decreases the transport of the aromatic amino acids, tyrosine and tryptophan, into the brain as can be predicted from our knowledge of transport competition through the blood–brain barrier [58].

The administration of branched-chain amino acids stimulates hepatic protein synthesis, reduces post injury catabolism and, therefore, improves nutritional status. Inadequate protein intake has a very deleterious effect on hepatic encephalopathy, nutritional status and clinical outcome of patients with liver disease [63]. Studies on inter-organ ammonia exchange in liver cirrhosis have shown that muscles may have a crucial role in ammonia detoxification. Nutritional guidelines have proposed that protein restriction should be avoided in patients with hepatic encephalopathy as protein requirement is even increased in cirrhotic patients [1].

Mesejio et al. [2] stated that in patients with liver cirrhosis and encephalopathy, metabolic impairment mimics a hypercatabolic state with increased protein and fat catabolism leading to depletion of protein and lipid reserves. Khanna and Gopalan [64] stated that there is ample evidence that patients with liver disease have an ongoing energy and protein catabolism.

Mesejio et al. [2] stated that protein restriction is not indicated in compensated cirrhosis. In acute encephalopathy temporary protein restriction may be needed, which should not last longer than 48 h. Better outcomes are obtained without severe protein restriction. Oral supplementation with branched-chain amino acids slows the progression of liver disease and improves survival and quality of life. On balance, branched chain amino acids (BCAAs) supplementation appears to be associated with decreased frequency of complications of cirrhosis and associated with improved metabolic profiles and clinical improvement of hepatic encephalopathy when prescribed as maintenance therapy [11]. Khanna and Gopalan [64] reported that the administration of BCCAs stimulates hepatic protein synthesis in patients with chronic liver disease with no reported toxic effects and added that the beneficial role of branched-chain amino acids supplementation in patients with chronic hepatic encephalopathy has been clearly documented. The FOXA (forkhead box A) proteins (FOXA1, FOXA2 and FOXA3) play a critical role in the development of the liver and they also regulate metabolism in adult hepatic tissue. Hepatic FOXA family of genes is differentially regulated by amino acids availability [46].

The rheological properties (meltability, penetration and oil separation) of processed cheese containing phenylalanine-free formula were higher than those of the control cheese without protein substitution. The melting value was increased with increasing percentage of the added protein substitution being highest in the processed cheese with 10% protein substitution. The higher melting value of processed cheese containing protein substitution may be due to its low casein and total protein contents [65].

The fat separation increased with increasing the added percentage of protein substitution in the formulae which could be attributed mainly to the nature of protein substitution in product. The protein matrix of Ras cheese (mainly casein) is able to retain fat and other components while protein substitution would weaken the matrix to be less able to retain the fat. The lowest penetration value was found in control cheese without protein substitution, while the highest was noticed with protein substitution cheese with 10% substitution followed by 5% then 2.5%. This means that control treatment was the firmest while 10% substitution was the softest. The difference in penetration value between control and treatments with protein substitution are mainly due to the cheese base used phenylalanine-free milk formula contained high soluble nitrogen that led to softer cheese [66].

In contrast, sensory evaluation of processed cheese indicate that cheese control without protein substitution was ranked the highest scores for different sensory attributes and highly accepted to panelists, followed by other treatments. Addition of 5% protein substitution of cheese improved the flavor, body and texture as well as cheese appearance of the resultant spreads which may make its rheological properties suitable for utilization by patients who are vomiting. It is difficult to give orally the usual quantity of BCAA free supplement particularly if the child is vomiting where the alternative is to use continuous nasogastric feeding [67]. On the other hand, increasing the ratio added protein substitution led to less acceptable products being lowest at 10% cheese base substitution.

In the present study, the improvement in brain pathology was best achieved in the mice fed on the processed cheese with 10% protein substitution. Branched- chain amino acids have been reported to improve fetal brain development in a rat model in which maternal PKU is induced by the inclusion of L-phenylalanine in diet [21]. Untreated patients with PKU

have been reported to have lower brain weights, changes in myelin structure, reductions in dendritic arborization and numbers of synaptic spines with selective vulnerable regions of the brain and disaggregation of brain ribosomes along with reduced polypeptide chain elongation on the polyribosomes of the brain. These effects are reversed by treatment of the mice with mixture of neutral amino acids including branched-chain amino acids [68]. The increased white matter water content has been suggested to reflect edema associated with gliosis within the tissue with defective myelination and status spongiosis [69]. There is dramatic increase in myelination after return of blood and brain phenylalanine to near normal control values [70]. The BCAAs increased in the brain as dietary casein level was increased. The magnitude of the responses in brain is approximately one-half the response in serum [71]. The ingestion of BCAAs causes rapid elevation of their plasma concentrations and increases their uptake by the brain [72]. Oral BCAA treatment has been applied to patients with stable hepatic cirrhosis based on the observation that liver failure produces elevated circulating levels of the aromatic amino acids and depressed concentrations of the BCAAs. Such changes increase brain concentrations of the aromatic amino acids, possibly stimulating the production of neurotransmitters and other biogenic amines that facilitate encephalopathy [73]. Supplying BCAAs is thus seen as a means to antagonize ArAA uptake into the brain and thus reduce the production of the biogenic amines derived from them. Indices of mental and motor function were significantly improved, and no adverse reactions were observed [74]. BCAA treatment reduced hospitalization, improved biochemical and pathophysiologic signs, and reduced anorexia [75].

Oral BCAA supplements have also been examined as a treatment for several neurologic diseases as bipolar subjects during periods of mania, on the presumption that this treatment will reduce brain tyrosine uptake and will slow catecholamine synthesis [76]. BCAAs have also been administered to patients with tardive dyskinesia. The application of oral BCAA therapy to this patient population followed from the observation that plasma phenylalanine concentrations were high in these patients, possibly causing abnormally high phenylalanine levels in the brain and adverse neuro chemical effects. The BCAAs have also been studied as a treatment for amyotrophic lateral sclerosis, BCAA administration has been hypothesized to restore GLU dehydrogenase enzyme activity, increase brain GLU disposal rate, and thereby diminish the neurotoxic effects of excessive extracellular GLU [77]. In the brain, GLU is an excitatory neurotransmitter; excessive extracellular levels can over stimulate neurons, causing them to die (excitotoxicity) [78].

5. Conclusion

The manufactured processed cheese in this study can be used as a therapeutic nutritional supplement rich in branched-chain amino acids. Best results and rheological evaluation were obtained with the 10% protein substitution in cheese while the best sensory score for flavor and appearance was for the 5% protein substitution in cheese. This may help in the implementation of dietary management in many medical and genetic disorders with liver and brain affection.

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